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Increased Expression of Fibronectin-Binding Proteins by Fluoroquinolone-Resistant *Staphylococcus aureus* Exposed to Subinhibitory Levels of Ciprofloxacin

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Bacterial adhesion, which plays an important role in *Staphylococcus aureus* colonization and infection, may be altered by the presence of antibiotics or/and antibiotic resistance determinants. This study evaluated the effect of fluoroquinolone resistance determinants on *S. aureus* adhesion to solid-phase fibronectin, which is specifically mediated by two surface-located fibronectin-binding proteins. Five isogenic mutants, derived from strain NCTC 8325 and expressing various levels of quinolone resistance, were tested in an in vitro bacterial adhesion assay with polymethylmethacrylate coverslips coated with increasing amounts of fibronectin. These strains contained single or combined mutations in the three major loci contributing to fluoroquinolone resistance, namely, *grlA*, *gyrA*, and *flqB*, which code for altered topoisomerase IV, DNA gyrase, and increased *norA*-mediated efflux of fluoroquinolones, respectively. Adhesion characteristics of the different quinolone-resistant mutants grown in the absence of fluoroquinolone showed only minor differences from those of parental strains. However, more important changes in adhesion were exhibited by mutants highly resistant to quinolones following their exponential growth in the presence of one-quarter MIC of ciprofloxacin. Increased bacterial adhesion of the highly quinolone-resistant mutants, which contained combined mutations in *grlA* and *gyrA*, was associated with and explained by the overexpression of their fibronectin-binding proteins as assessed by Western ligand affinity blotting. These findings contradict the notion that subinhibitory concentrations of antibiotics generally decrease the expression of virulence factors by *S. aureus*. Perhaps the increased adhesion of *S. aureus* strains highly resistant to fluoroquinolones contributes in part to that emergence in clinical settings.

Bacterial adhesion to host cells or tissues is an important step in the initiation of bacterial infection. This process also plays an important role in bacterial colonization of medical devices coated with various cellular and extracellular host components. Several studies have shown that *Staphylococcus aureus* expresses specific surface proteins called adhesins (9, 10, 17, 36) that allow the organism to interact specifically with a number of plasma or extracellular matrix proteins present on normal tissues or adsorbed on biomedical devices. The most important host proteins promoting adhesion of *S. aureus* are fibronectin (16, 54, 55, 57, 58), fibrinogen (14, 16, 27), collagen (36–39), vitronectin (24), laminin (16, 26), thrombospondin (15), bone sialoprotein (61), and elastin (60). Cloning and sequencing of the genes coding for fibronectin-binding (8, 11, 12, 20, 25), fibrinogen-binding (27), and collagen-binding (37, 40) proteins of *S. aureus* have recently been reported. An important aspect of these molecular studies was the demonstration of the functional significance of such adhesins by the production of specific mutants expressing defective attachment to their respective host proteins in vitro and in vivo (11, 12, 27, 38, 59).

The fibronectin-binding activity is due to two distinct cell wall-associated proteins called fibronectin-binding proteins (FnBPs), which are encoded by closely linked but separately transcribed genes, *fnbA* and *fnbB* (8, 11, 20). A recent study showed that both *fnb* genes must be inactivated to eliminate bacterial interactions with fibronectin (11).

The fluoroquinolones are known to act on DNA gyrase, an essential topoisomerase composed of two A (GyrA) and two B (GyrB) subunits (19, 41, 50). Alterations in specific domains of both the *gyrA* and the *gyrB* genes (encoding GyrA and GyrB, respectively) have been shown to cause fluoroquinolone resistance in *Escherichia coli* and other gram-negative and gram-positive bacteria, identifying DNA gyrase as an important drug target in these different bacterial species (18).

In *S. aureus*, expression of fluoroquinolone resistance is mediated by at least three mechanisms acting separately or in concert: (i) specific deduced amino acid substitutions in GyrA (19, 31, 41, 50, 51); (ii) increased *norA*-mediated efflux of fluoroquinolones, associated with the *flqB* resistance locus (21, 30, 32, 34, 50); and (iii) specific deduced amino acid substitutions in GrlA, one of the subunits of topoisomerase IV (6, 7, 31, 53, 62). Recent molecular and genetic studies of both clinical and laboratory isolates of *S. aureus* indicate that first-step mutations leading to low levels of fluoroquinolone resistance occur in *grlA*, which codes for an altered topoisomerase IV, and do not involve mutations in the *gyrA* or *gyrB* genes. Furthermore, the Ser84Leu mutation in *gyrA* which confers one-step high-level quinolone resistance in *E. coli* is unable to cause resistance in *S. aureus* without the concomitant presence of *grlA* mutations (31). Taken together, these data indicate that topoisomerase IV and not gyrase is the primary target of current fluoroquinolones in *S. aureus*. Furthermore, high levels of fluoroquinolone resistance in *S. aureus* often involve the oc-

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TABLE 1. Bacterial strains used in the study[a]

S. aureus strain	Genotype	Properties	Origin or reference	MIC (μg/ml) of ciprofloxacin
ISP794	8325 *pig-131*	Nov^s parent	49	0.25
MT5	8325 *nov-142 hisG15 pig-131*	Nov^r parent	53	0.25
SS1	MT5 *gyrA*	Silent mutation	This study	0.25
MT5224c4	MT5 *flqA(grlA542)*	Mutation in GrlA (topoisomerase IV)	31, 53	2.0
MT23142	ISP794 *flqB* Ω*(chr::*Tn*916)1108*	Increased *norA*-mediated quinolone efflux	32	1.0
EN1252a	MT 5 *flqA(grlA542) gyrA*Ω*1051*(Erm) Nov$^+$	Double mutation in *grlA* and *gyrA*	This study	32.0
MT1222	ISP794 *grlA flqB gyrA*	Increased *norA*-mediated quinolone efflux plus mutations in *grlA* and *gyrA*	31, 53	64.0
DU5723	Δ*spa-600*::EtBr^r	Protein A-deficient mutant of 8325-4	35	ND
DU5883	*fnbA*::Tc^r *fnbB*::Em^r	Mutant of 8325-4 defective in FnBPA and FnBPB	11	ND
DU5883(pFNBA4)	*fnbA*::Tc^r *fnbB*::Em^r (pFNBA4)	Mutant of 8325-4 defective in FnBPA and FnBPB with multicopy plasmid overexpressing FnBPA	11	ND
DU5883(pFNBB4)	*fnbA*::Tc^r *fnbB*::Em^r (pFNBB4)	Mutant of 8325-4 defective in FnBPA and FnBPB with multicopy plasmid overexpressing FnBPB	11	ND

[a] Abbreviations: Nov^s, novobiocin susceptible; Nov^r, novobiocin resistant; ND, not determined; EtBr^r, ethidium bromide resistant; Tc^r, tetracycline resistant; Em^r, erythromycin resistant.

currence of combined mutations in *grlA* and *gyrA*, leading to altered topoisomerase IV and DNA gyrase (31).

Within a brief period after the introduction of the fluoroquinolones into clinical practice, strains of *S. aureus* expressing resistance to these compounds were detected, in particular among methicillin-resistant isolates, which frequently exhibit a multiresistance pattern to several unrelated antimicrobial agents (52). The molecular mechanisms explaining colonization of compromised patients and medical staff in an endemic and sometimes epidemic way (1, 28, 44) by multiresistant strains are poorly understood. In particular, we lack detailed molecular studies of adhesion to mucous layers and extracellular matrix proteins of antibiotic-resistant strains of *S. aureus* compared to antibiotic-susceptible organisms.

The aim of this report is to describe the overexpression of FnBPs in genetically characterized strains of *S. aureus* that express specific single or multiple mutations conferring resistance to fluoroquinolones. To simplify this analysis, we analyzed isogenic quinolone-resistant mutants derived from methicillin-susceptible parent strains. The addition of subinhibitory concentrations of ciprofloxacin to the growth medium of some of the quinolone-resistant mutants was the most effective factor for triggering overproduction of the fibronectin adhesins.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in the study are listed in Table 1.

Construction of strains EN1252a and SS1. To construct a strain having both *gyrA* and *grlA* resistance mutations, strain MT5224c4 [*grlA542* (Ser80Phe) *nov-142*] was used as a recipient for transformation with high-molecular-weight DNA prepared from strain MT1293 [*gyrA*Ω(*chr*::Tn*551*)*1051*]. Transformants were plated on erythromycin (20 μg/ml) agar to select for Tn*551* and scored for increases in the MIC of ciprofloxacin. Twenty-three (55%) of 42 transformants tested had increases in the MIC of ciprofloxacin from 2 to 32 μg/ml, a linkage consistent with that previously reported between *gyrA* and Ω(*chr*::Tn*551*)*1051* (31). EN1252a (*grlA542 gyrA nov-142*) is one of the highly ciprofloxacin-resistant transformants.

To construct a strain with a silent *gyrA* mutation in the absence of a *grlA* mutation, wild-type strain ISP794 was used as recipient for transformation with DNA prepared from strain MT3229 [*gyrA* (Ser84Leu) *nov-142*] (31). Transformants were plated on novobiocin (10 μg/ml) agar to select for *nov*. Because *gyrA* has no resistance phenotype in the absence of *grlA* mutations, SS1 and other transformants were purified and additionally tested to determine the presence of a *gyrA* mutation by a second transformation with DNA from strain MT4172 [*grlA541* (Ser80Phe) Ω(*chr*::Tn*917lac*)*2*] (53). In this transformation, a *grlA* mutation is introduced by selection on erythromycin agar for the linked Tn*917lac* in a procedure identical to that previously described in which the increment in ciprofloxacin resistance of *grlA* transformants of recipients containing *gyrA* mutations substantially exceeds that attributable to *grlA* alone in control transformations (31). SS1 (*gyrA nov-142*) was documented to have a silent *gyrA* mutation by this method.

Determination of ciprofloxacin susceptibility. The MICs of ciprofloxacin for fluoroquinolone-resistant or -susceptible strains were determined by a macrodilution method using Mueller-Hinton broth (Difco, Detroit, Mich.) and a standard inoculum of 10^6 CFU/ml (29).

Bacterial adhesion assay. Purified human fibronectin (Chemicon, Temecula, Calif.) was dissolved in phosphate-buffered saline (PBS) at 1 mg/ml and stored at −70°C. The concentration was measured spectrophotometrically using an extinction coefficient of $E_{280} = 1.28$. The attachment properties of *S. aureus* strains were measured using a previously described adhesion assay with polymethylmethacrylate (PMMA) coverslips coated in vitro with purified fibronectin (11, 12, 58). To optimize adsorption of fibronectin from concentrations ranging from 0.5 to 2 μg/ml, the PMMA coverslips were precoated with gelatin (1 mg/ml) as previously described (11, 12). After being rinsed in PBS, they were incubated in duplicate for 60 min at 37°C with three different concentrations (0.5, 1, and 2 μg/ml) of fibronectin in PBS and then rinsed in PBS as previously described (11, 12). PMMA surfaces were shown to be coated in a dose-dependent manner with fibronectin ranging from 88 to 296 ng per coverslip (60 to 206 ng/cm^2) when radiolabeled fibronectin (by reductive methylation with sodium boro[^{3}H]hydride [48, 56]) was used.

The adhesion characteristics of the different strains of *S. aureus* were evaluated by incubating the protein-coated coverslips with 10^7 CFU of washed cultures of late-logarithmic-phase cells metabolically radiolabeled with [^{3}H]thymidine during growth for 5 h at 37°C in Mueller-Hinton broth as previously described (11, 12, 58). When the influence of subinhibitory concentrations of fluoroquinolones on bacterial adhesion was tested, ciprofloxacin was added to the 5-h cultures of quinolone-resistant or -susceptible strains of *S. aureus* at a concentration of one-quarter MIC for each strain.

Control experiments verified that all strains grown either in antibiotic-free or ciprofloxacin-containing medium contained a high percentage (>95%) of cell-associated [^{3}H]thymidine precipitable by trichloroacetic acid. Compared with that in parental strain ISP794, the range of [^{3}H]thymidine incorporated into the other strains growing in antibiotic-free medium was 57 to 147%. For all strains, the label was correlated with the number of viable counts, which varied from 87 to 120% compared with that in strain ISP794. After growth in ciprofloxacin-containing medium, the different strains showed an average 50% reduction in both radioactive and CFU counts.

The protein-coated coverslips were incubated with radiolabeled bacteria for 60 min at 37°C in PBS with 1 mM Ca^{2+} and 0.5 mM Mg^{2+} supplemented with 5 mg of human albumin per ml, which prevented nonspecific adhesion of *S. aureus* as previously described (11, 12, 58). At the end of the attachment period, the fluids containing unbound bacteria were removed, the coverslips were rinsed, and radioactivity on the coverslips was counted as previously described (58). To compare under normalized conditions adhesion of different strains whose cell-associated radioactive contents differed slightly, the amount of radioactivity remaining on the coverslips divided by the amount of radiolabeled bacteria initially added to the system (expressed as a percentage) was first evaluated. Control experiments verified that the percentage of radiolabeled bacteria attached to the coverslips was constant when the inoculum size varied from 2×10^6 to 2×10^7 CFU/ml. For each strain, the percentage of attached radiolabeled bacteria was used to estimate the number of adherent CFU per coverslip nor-

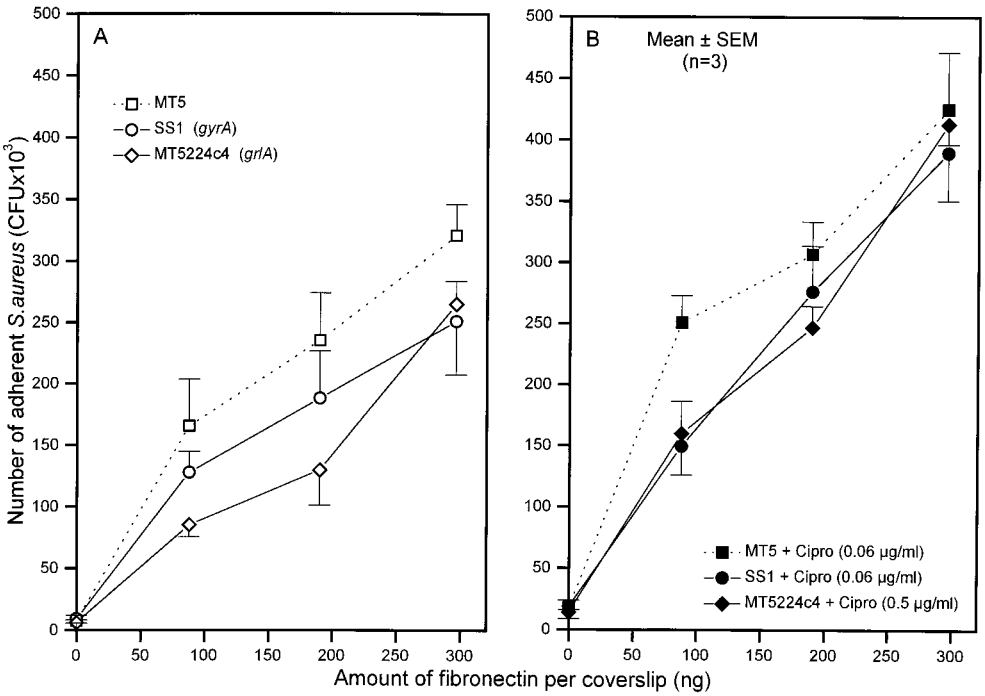

FIG. 1. Adhesion to fibronectin-coated coverslips of *S. aureus* strains grown in the absence (A) or presence (B) of the indicated concentrations of ciprofloxacin (Cipro), which are equivalent to one-quarter MIC for each strain.

malized to a fixed inoculum of 10^7 CFU/ml. For each experiment, gelatin-coated PMMA coverslips were used as controls of adhesion to fibronectin-coated surfaces (11, 12).

Each experiment was performed at least three times, and the results were expressed as means ± standard errors. The nonparametric Wilcoxon test was performed to evaluate the significance of differences either between parental and any mutant strain, or between exposure or no exposure to ciprofloxacin for each strain. The adhesion profiles of two different strains or of one strain both exposed and not exposed to ciprofloxacin were considered significantly different from each other when all increases or decreases accumulated for the three coating concentrations of fibronectin yielded P values of <0.05 with a two-tailed significance level. This simplified approach was chosen because the fibronectin dose-adhesion response data did not at all meet the criteria for curve fitting that were required for a direct computerized test of the statistical differences.

SDS-PAGE and Western ligand affinity blotting. Bacterial protein extracts from each strain of *S. aureus* were prepared by growing organisms without shaking in 100 ml of Mueller-Hinton broth, supplemented or not with one-quarter MIC of ciprofloxacin for each strain for 5 h at 37°C. After two washes in PBS, the bacterial cultures were concentrated and lysed in 1.5 ml of PBS with 1 mM Ca^{2+} and 0.5 mM Mg^{2+} containing 20 μg of lysostaphin (Ambicin; Applied Microbiology, Inc., Brooklyn, N.Y.) per ml, 20 μg of DNAse per ml, and a mixture of proteinase inhibitors (Complete; Boehringer, Mannheim, Germany) for 15 min at 37°C. The lysate was centrifuged at 2,000 × *g* for 10 min, and the supernatant was retained. After adjustment of the concentrations of extracted proteins from each strain by the BCA method (Pierce), protein amounts equivalent to 24 μg per strain (except when indicated) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4 to 15% acrylamide gradient (23). Proteins were transferred to an Immobilon membrane (Millipore) by using a liquid transblot system (BioRad). The membrane was blocked in 10 mM Tris/HCl (pH 8.0)–500 mM NaCl–0.1% Tween 20 (Fluka) (TBST) containing 2% bovine serum albumin. FnBPs were detected by Western ligand affinity blotting by incubation with pure human fibronectin (30 μg/ml) in TBST. The membrane was rinsed several times in TBST and incubated with monoclonal antibody MAb-1936 raised against the N terminus of fibronectin (1:5,000; Chemicon) followed by peroxidase-conjugated anti-mouse immunoglobulin G (1:10,000 dilution; Amersham). Detection was by enhanced chemiluminescence (Amersham). Strain-specific quantitative differences in the content of FnBPs were estimated by scanning (Molecular Dynamics) the bands corresponding to native FnBPs, which were densitometrically analyzed with an imaging system (MD Image Quant). The locations of native FnBPs were assessed by running in parallel lysostaphin protein extracts of strains DU5883(pFNBA4) and DU5883(pFNBB4), which contain multicopy plasmids expressing large amounts of FnBPA and FnBPB, respectively.

RESULTS

Adhesion characteristics of *S. aureus* quinolone-resistant mutants grown in the absence of fluoroquinolone. The adhesion profiles of the different mutants derived from either the parental novobiocin-resistant strain MT5 or the novobiocin-susceptible strain ISP794 are shown in Fig. 1 and 2, respectively. Bacterial attachment increased dose dependently as a function of the amount of adsorbed fibronectin per coverslip. When the mutants were grown in the absence of fluoroquinolone, they attached normally to fibronectin-coated coverslips, and only two of them exhibited significant differences with their respective quinolone-susceptible parents. The *grlA* single mutant (strain MT5224c4) attached significantly less ($P < 0.05$) than its respective novobiocin-resistant parent (Fig. 1A), unlike the *grlA gyrA* double mutant (strain EN1252a), which attached significantly more ($P < 0.05$) than its novobiocin-susceptible parent (Fig. 2A). Two other mutants, namely, strain MT23142 *flqB* (Fig. 2A), which exhibits increased levels of expression of *norA*, and the *gyrA* single-mutant strain SS1 (Fig. 1A), which does not cause ciprofloxacin resistance by itself in *S. aureus* (31), exhibited adhesion profiles similar to those of their respective parents. This was also the case for the highly resistant strain MT1222 (Fig. 2A), which had been selected by serial passage on increasing concentrations of norfloxacin to generate multiple mutations (31, 53). This strain was previously shown to contain mutations in *grlA* (Glu116), *gyrA* (Leu84), and the *flqB* resistance locus associated with overexpression of *norA*.

Finally, the increased-adhesion profile of the novobiocin-resistant parent (strain MT5) (Fig. 1A) over the novobiocin-susceptible parent (strain ISP794) (Fig. 2A) did not reach statistical significance.

Adhesion of *S. aureus* quinolone-resistant mutants grown in the presence of subinhibitory levels of ciprofloxacin. Adhesion

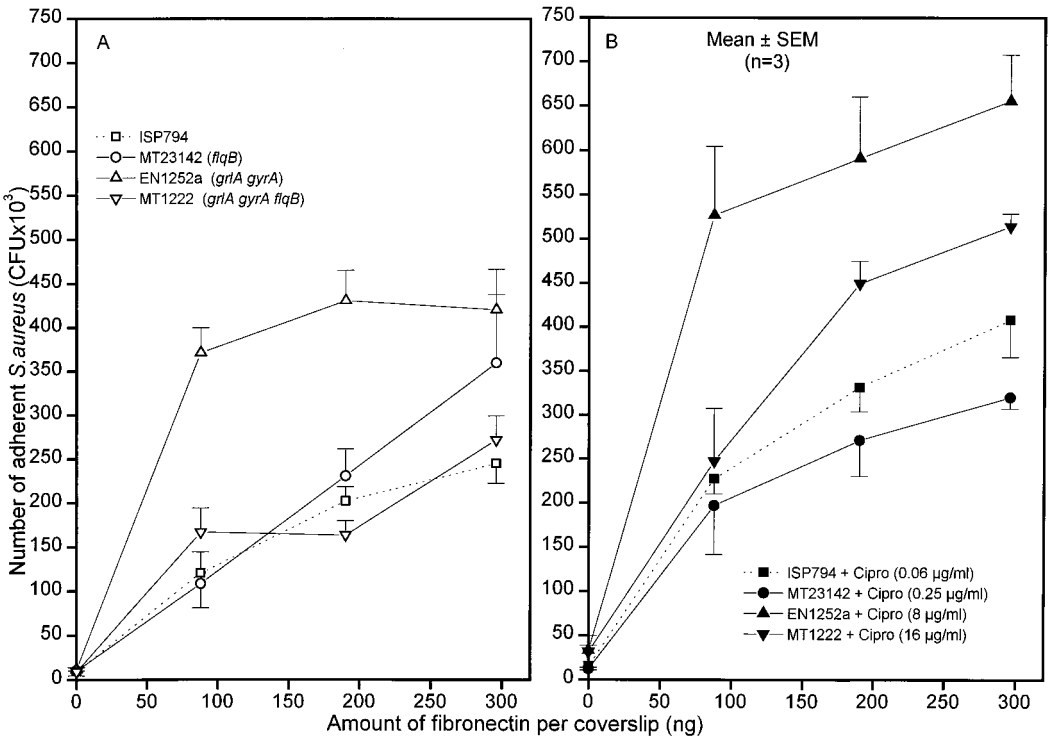

FIG. 2. Adhesion to fibronectin-coated coverslips of *S. aureus* strains grown in the absence (A) or presence (B) of the indicated concentrations of ciprofloxacin (Cipro), which are equivalent to one-quarter MIC for each strain.

of the different mutants and parental strains grown in the presence of subinhibitory concentrations of ciprofloxacin equivalent to one-quarter MIC for each strain (Fig. 1B and 2B) was tested in parallel to that of the same strains grown in antibiotic-free medium (Fig. 1A and 2A). The adhesion profiles of the *gyrA* single mutant (strain SS1) and the *grlA* single mutant (strain MT5224c4) grown in the presence of 0.06 and 0.25 μg of ciprofloxacin per ml, respectively, exhibited moderate but significant ($P < 0.05$) increases over those of organisms grown in the absence of the fluoroquinolone (Fig. 1A and 1B). In contrast to the other mutants, strain MT23142 *flqB*, associated with overexpression of *norA*, showed no significant increase in adhesion by growth in the presence of 0.25 μg of ciprofloxacin per ml (Fig. 2B). This was also the case for the parental strain MT5, whose increase was not significant after incubation with 0.06 μg of ciprofloxacin per ml.

The highest values in adhesion were recorded with the two more highly resistant strains of *S. aureus* that yielded combined *grlA* and *gyrA* mutations (Fig. 2B) (31). The *grlA gyrA* double-mutant strain EN1252a and the multiple-mutant strain MT1222 exhibited significant ($P < 0.02$) increases in attachment to fibronectin after growth in the presence of 8 and 16 μg of ciprofloxacin per ml, respectively, compared to growth in antibiotic-free medium (Fig. 2A and 2B). After growth in the presence of subinhibitory levels of ciprofloxacin, the adhesion profile of strain MT1222 increased by an average of twofold, exceeding that of parental strain ISP794 (Fig. 2B).

Control experiments verified that the observed differences in the adhesion profiles of highly quinolone-resistant strains were not due to increased cell-to-cell interactions. Viable counts of suspensions of radiolabeled bacteria performed before and after sonication showed <20% variations in all strains, regardless of their prior growth conditions in the presence or absence of one-quarter MIC of ciprofloxacin. These data ruled out the occurrence of significant clumping in some of the strains tested for adhesion. Finally, we also assayed unlabeled bacterial suspensions for adhesion to fibronectin by performing viable counts of organisms released from the coverslips by trypsinization. Comparison of bacterial adhesion of the parental strain ISP794 with the *grlA gyrA* double-mutant strain EN1252a assessed that the highly quinolone-resistant mutant was more adhesive than its parent, in particular after growth in the presence of one-quarter MIC of ciprofloxacin (data not shown).

Expression of FnBPs by *S. aureus* quinolone-resistant mutants. Western ligand affinity blotting was performed on lysostaphin extracts of the different *flq* mutants and parental strains of *S. aureus* (Fig. 3). The locations of native FnBPs or their high-molecular-weight truncated derivatives were determined by running in parallel protein extracts of strains DU5883(pFNBA4) and DU5883(pFNBB4), which contain multicopy plasmids expressing high amounts of FnBPA and FnBPB, respectively (data not shown). Because FnBPA and FnBPB have closely related electrophoretic mobilities on SDS-PAGE, they were not discriminated from each other on the Western ligand affinity blots performed with the *flq* mutants and parental strains of *S. aureus*. Figure 3 shows that parental strains ISP794 and MT5 exhibited a pattern of FnBPs similar to that previously described with the closely related strain 8325-4 (11), namely, a faint band of 180- to 200-kDa native proteins (band I) plus another band of 130- to 140-kDa proteins (band II) likely derived from native FnBPs by proteolytic degradation. Bands I and II were identified as native and truncated FnBPs, respectively, because they were overexpressed in strains bearing individual *fnb* genes cloned on multicopy plasmids and selectively missing in the *fnbA fnbB* double-mutant strain DU5883, which is defective for FnBP

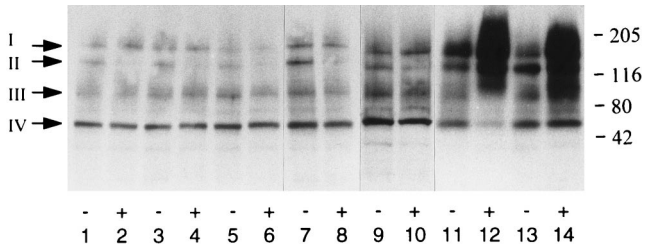

FIG. 3. Visualization of FnBPs by Western ligand affinity blots in bacterial lysates of quinolone-resistant and -susceptible strains of *S. aureus*. Positions of protein size markers (in kilodaltons) are shown at right. Bands I and II correspond to native and truncated FnBPs, respectively. Band III is an unknown 88-kDa component unrelated to FnBPs. Band IV is identified as protein A. See text for details. Lanes 1 and 2, ISP794; lanes 3 and 4, MT5; lanes 5 and 6, SS1 *gyrA*; lanes 7 and 8, MT23142 *flqB*; lanes 9 and 10, MT5224c4 *grlA*; lanes 11 and 12, EN1252a *grlA gyrA*; lanes 13 and 14, MT1222 *grlA gyrA flqB*; lanes 1, 3, 5, 7, 9, 11, and 13, growth without ciprofloxacin (−); lanes 2, 4, 6, 8, 10, 12, and 14, growth with one-quarter MIC of ciprofloxacin (+).

production (11) (data not shown). In contrast, band III showed neither increase in the strains complemented with *fnb* genes on multicopy plasmids nor decrease in the *fnbA fnbB* double-mutant DU5883 (data not shown). Thus, band III is an unidentified 88-kDa component of *S. aureus* unrelated to FnBPs whose location and function are unknown. This band will not be discussed further because its intensity is not influenced by incubation with subinhibitory concentrations of ciprofloxacin or by the presence of any fluoroquinolone resistance determinant. Finally, band IV, which is a 56-kDa protein, was easily identified as protein A, because it is absent from lysostaphin extracts of a site-specific protein A-defective mutant (DU5723) (35) derived from strain 8325-4 (data not shown).

No significant difference was observed in the intensities of native FnBPs (band I) of parental strains (lanes 1 through 4) and each category of single *flq* mutants (lanes 5 through 10) of *S. aureus* grown in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of one-quarter MIC of ciprofloxacin. A common characteristic of all these strains was to reveal a low content in native (band I) and truncated (band II) FnBPs. In contrast to band I, whose intensity was not significantly influenced by growth in the presence or absence of one-quarter MIC of ciprofloxacin, the intensity of band II seemed to be selectively decreased after growth in the quinolone-containing medium. The mechanisms explaining the decreased amount of truncated derivative band II are unknown.

A striking feature of the *grlA gyrA* double mutant (strain EN1252a, lane 12) and the multiple mutant (strain MT1222, lane 14) also carrying the *norA*-overexpressing *flqB* mutation was their strongly increased content of FnBPs, specifically after growth in the presence of 8 and 16 μg of ciprofloxacin per ml, respectively. More contrasting results were obtained after growth of either mutant strain in antibiotic-free medium. Whereas the double mutant EN1252a (lane 11) expressed a higher content in band I than in band II, the opposite seemed to be true for the triple mutant MT1222 (lane 13). The reasons for these differences are unknown.

To more precisely evaluate the quantitative increase in FnBP production by strains EN1252a and MT1222 compared to the other strains, serial twofold dilutions of the protein extracts of these highly producing strains were analyzed by SDS-PAGE and Western ligand affinity blotting. Figure 4 shows that the amount of native FnPBs (band I, Fig. 3) quantified by densitometric analysis was strictly proportional to the amount of protein extracts loaded for SDS-PAGE. Strain EN1252a grown in the presence of ciprofloxacin had a twofold-

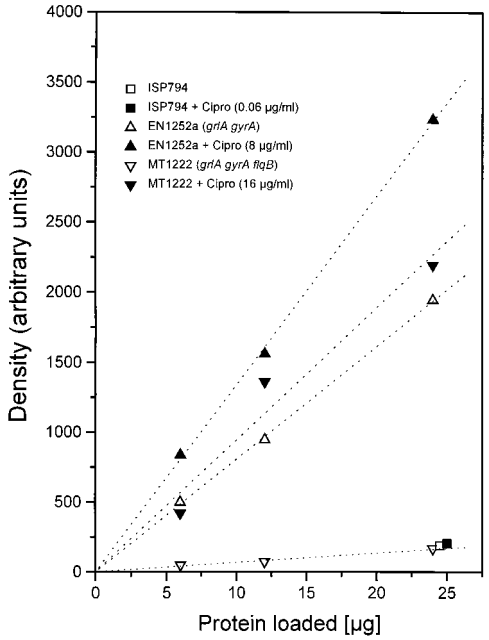

FIG. 4. Quantitative densitometric analysis of native FnBP expression in *S. aureus* strains grown in the absence or presence of the indicated concentrations of ciprofloxacin (Cipro), which are equivalent to one-quarter MIC for each strain.

higher content in band I than after growth in antibiotic-free medium. A much larger increase in band I was seen with strain MT1222 after growth in ciprofloxacin-containing than after growth in antibiotic-free medium, which represented a >10-fold increase in the production of native FnBPs. In contrast to band I, band II was not significantly increased by growth of the highly quinolone-resistant mutants in the presence of one-quarter MIC of ciprofloxacin.

Taken together, these data indicate that subinhibitory levels of ciprofloxacin in the growth medium greatly stimulated the production of fibronectin adhesins in *S. aureus* strains highly resistant to fluoroquinolones. The highly increased production of FnBPs by these mutants grown in the presence of sub-MIC levels of ciprofloxacin likely explained their higher level of adhesion to fibronectin-coated coverslips.

DISCUSSION

This study shows that expression of fibronectin adhesins by some highly quinolone-resistant mutants of *S. aureus* can be increased by growth in the presence of subinhibitory levels of ciprofloxacin. The increased expression of fibronectin adhesins by the quinolone-resistant organisms leads to increased attachment of the bacterial cells to immobilized fibronectin in an in vitro model. Further clinical and epidemiological studies are warranted to evaluate the potential contribution of fluoroquinolones and fluoroquinolone resistance genes to the colonization by *S. aureus* of implanted biomedical devices and host tissues. That this antibiotic-promoted increase in the adhesion properties of highly quinolone-resistant mutants of *S. aureus* may contribute to the emergence of organisms expressing increased levels of antibiotic resistance is an attractive but as yet unproven hypothesis.

A large number of studies have described the beneficial effects of antibiotics, including fluoroquinolones, even when these agents reached subinhibitory concentrations during ther-

apy or in various in vivo or in vitro experimental conditions. These studies (reviewed in references 45 through 47) indicated that exposure to sub-MIC levels of fluoroquinolones and some other antibiotics could alter bacterial cell morphology and adhesion (2) and lead to increased phagocytosis and intracellular killing of various bacterial species, including *S. aureus* (5). These experimental results led to the widely accepted notion that subinhibitory concentrations of antibiotics tend to decrease expression of virulence factors by *S. aureus* and other bacterial species. Very few reports have challenged this concept by showing that sub-MIC levels of antibiotics could increase expression of bacterial virulence factors. One recent study provided evidence for increased α-toxin production by *S. aureus* growing in the presence of sub-MIC levels of nafcillin (22).

Studies evaluating the effects of sub-MIC levels of antibiotics on specific adhesins of *S. aureus* are quite limited because most of these adhesins have been only recently characterized. Various inhibitory or promoting effects on adhesin expression and adhesin-mediated attachment of *S. aureus* have been reported for either FnBPs (42, 43) or collagen-binding protein(s) (3). These variable responses to subinhibitory antibiotics were likely influenced by the mode of action and bacterial targets of the different agents evaluated in a variety of in vitro assays. As far as we know, there has been no systematic study correlating increase in bacterial adhesion with acquisition of antibiotic resistance determinants and the presence of subinhibitory levels of fluoroquinolones.

The most striking effects of sub-MIC concentrations of ciprofloxacin were seen in the most highly resistant strains, for which one-quarter MIC is 8 to 16 μg per ml, a concentration that is somewhat higher than the usual peak concentration in serum of 3 to 5 μg/ml with human use. Higher concentrations of ciprofloxacin are, however, achieved at some body sites, including within macrophages and phagocytes and in urine and the gastrointestinal tract with oral dosing. Subtler effects may be seen at lower concentrations. It is possible that the role of the dual *grlA* and *gyrA* mutation in our resistant mutant is nonspecific, providing a sufficient level of resistance to allow cell survival at concentrations of ciprofloxacin needed to produce an effect on FnBP expression that is demonstrable in our measurements by Western analysis and whole-cell adherence assay. Application of more sensitive assays of *fnbA* and *fnbB* expression, which are under development, may allow a clearer demonstration of an effect of ciprofloxacin at even lower therapeutic concentrations.

The molecular mechanisms of the regulatory effects of antibiotics on adhesin expression are unknown and warrant further investigations. In this study, the contribution of each fluoroquinolone resistance determinant to the modulation of fibronectin adhesins could be precisely studied by comparing five isogenic mutants of strain NCTC 8325 that yield single or combined mutations conferring low or high levels of quinolone resistance. These different loci controlling expression of quinolone resistance were shown to have different effects on bacterial adhesion. On one hand, we could not detect any significant influence of an increased *norA*-mediated efflux of ciprofloxacin on the bacterial adhesion mechanisms of the *flqB* mutant strain MT23142. Furthermore, the presence of sub-MIC levels of ciprofloxacin in the growth medium of this *norA*-overexpressing mutant did not change its attachment characteristics to fibronectin. Two other single mutants, having alterations in either of the *grlA* or *gyrA* loci, exhibited minor increases in bacterial adhesion after growth in ciprofloxacin-containing compared to antibiotic-free medium. In comparison to the low-level responses exhibited by single mutants, the two mutants yielding combined *grlA* and *gyrA* mutations markedly increased their attachment to fibronectin after growth in the presence of subinhibitory levels of ciprofloxacin. Whereas one of these highly quinolone resistant strains (EN1252a) is a well-characterized, genetically engineered *grlA gyrA* double mutant, the other strain (MT1222) is a naturally resistant mutant selected by serial passage on increasing concentrations of norfloxacin to generate multiple mutations, three of which (*grlA*, *gyrA*, and *flqB*) have been defined. A major link between both strains is that strain MT1222 was the donor of the *gyrA* (Ser84Leu) mutation. In contrast, strains MT1222 and EN1252a have different mutations in their *flqA* locus, namely, *grlA* (Ala116Glu) and *grlA* (Ser80Phe), respectively, plus the presence in the former but not the latter strain of the *flqB norA*-overexpressing resistance locus. It is possible that these differences in the *grlA* and *flqB* loci of strain EN1252a versus MT1222 account for their different adhesion characteristics after growth in antibiotic-free medium.

The specific mechanisms by which combined mutations in *grlA* and *gyrA* effect increases in FnBPs is as yet undefined but might relate to several potential mechanisms: (i) alterations in DNA supercoiling, which are known to affect the expression of many genes; (ii) induction of complex response systems such as the SOS response; or (iii) induction or repression of other global regulators such as *agr*, *sar*, or *xpr* (4, 13, 33). Previous studies documented that expression of FnBP(s) is under the control of *agr*, which is responsive to phase growth. Further molecular studies with mutants specifically defective in each of these global response systems are planned to evaluate their respective contributions to increased FnBP expression in quinolone-resistant mutants. Finally, the striking effects of ciprofloxacin to enhance further the expression of FnBPs in such mutants might also result from less specific mechanisms. Possibly, ciprofloxacin has additional actions on the bacterial cell at concentrations that can be tested only in mutants in which the interaction of the drug with the targets is blocked by dual mutations in *grlA* and *gyrA*. Further studies will be needed to evaluate these possibilities.

Studies in progress in our laboratory indicate that attachment to surface-bound fibronectin of a number of methicillin-susceptible or methicillin-resistant clinical isolates of *S. aureus* expressing high levels of fluoroquinolone resistance may also be promoted by growth in the presence of sub-MIC levels of ciprofloxacin. Further studies will need to be undertaken to evaluate the molecular mechanisms and epidemiological relevance of these microbiological observations.

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